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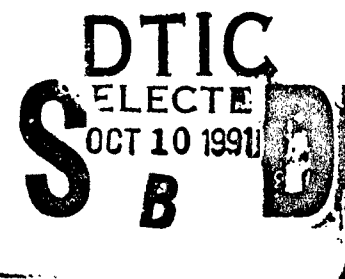
MECHANISMS OF ACTION OF CLOSTRIDIAL NEUROTOXINS ON DISSOCIATED
MOUSE SPINAL CORD NEURONS IN CELL CULTURE

ANNUAL REPORT

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The mechanism of action of tetanus toxin was investigated using the in vitro system of dissociated mouse spinal cord neurons in culture. Monoclonal antibodies directed against various epitopes on the toxin molecule were investigated with regard to their ability to neutralize the convulsant effects of the toxin. Of the antibodies tested, neutralizing antibodies were found that were directed against the binding subunit (fragment C) of the toxin as well as the heavy chain portion of the nonbinding subunit (fragment B). These findings suggest that the active domain of the toxin molecule resides on the B subunit.

Other experiments utilizing agents to potentiall antagonize tetanus toxin revealed that chloramine was toxic to the neurons, ammonium chloride produced convulsant activity itself, Concancavalin A appeared to block synaptic transmission itself and cGMP failed to reverse the effects of the toxin.

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

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STATEMENT OF PROBLEM UNDER STUDY

The research conducted during the period of this annual report continued the investigations of structure function relationships of tetanus toxin. As outlined in the specific aims of the original proposal, investigations were conducted:

1. To use monoclonal antibodies directed against selected domains of the tetanus toxin molecule to determine which region is the active moitey and which portion of the B subunit is the active subunit.

2. To study the ability of various agents including lectins, ammonium chloride, methylamine hydrochloride and chloroquine to anatagonize the convulsant action of tetanus toxin on the spinal cord neurons in culture.

BACKGROUND AND INTRODUCTION

The clostridial neurotoxin tetanus toxin is one of the most potent substances known. After binding to peripheral nerve endings the toxin is then transported retrograde in axons (Price et al., 1975), reaching the motoneurons of the ventral horn of the spinal cord. This unique toxin then moves transsynaptically to reach the presynaptic nerve terminals where it acts to block release of neurotransmitter, preferentially affecting inhibitory systems to produce the characteristic clinical picture of spinal disinhibition. Recent studies using in vitro systems and artificial membranes have extended the knowledge regarding toxin interactions with cell membranes. It is now thought that toxin action involves three steps, a binding step, a complicated membrane interaction or internalization step and then the step actually producing blockade of neurotransmitter release (Schmitt et al., 1981; Critchley et al., 1985). The locus and mechanism of action for blockade of neurotransmitter release by tetanus toxin is not known.

The basic structure of tetanus toxin is fairly well understood (Robinson and Hash, 1982). Recently the nucletide sequence has been partially determined (Eisel et al., 1986). Initially produced as a single chain of about 150,000 molecular weight, the toxin is then "nicked" by bacterial proteases to form a two chain structure composed of a heavy chain of about 96,000 daltons joined by a disulfide bond to a light chain of about 47,000 daltons (Matsuda and Yoneda, 1975; Helting and Zwisler, 1977). This nicking now appears to be necessary for maximum toxin activity (Bergey et al., 1989). This two chain structure is similar in design to that of other cellular toxins (e.g. botulinum toxin, diptheria toxin). The carboxyterminal of the heavy chain contains the binding region of the toxin molecule. Experimental cleavage with papain can produce a 50,000 molecular weight subunit of the heavy chain that contains the binding domain; this subunit has been termed Fragment C (Figure 1).

Fragment C alone is nontoxic (Helting and Zwisler, 1977). The complementary portion, Fragment B, contains the entire light chain and the remaining heavy chain region. Fragment B also is of low toxicity but this presumably is due to the fact that the absence of the binding region limits toxin entry into the neurons. Indeed, recent studies by Penner et al. (1986) utilizing whole cell patch clamp techniques to introduce a B fragment preparation into the cell interior of adrenal chromaffin cells demonstrate that the B fragment preparation can block exocytosis in these cells. The aminoterminus of the heavy chain (i.e. that portion in Fragment B) can form channels in artificial membranes; it has been proposed that this region is responsible for internalization of the toxin (Boquet and Duflot, 1982). Because the heavy chain alone is nontoxic and implicated in channel formation, speculation is that the light chain of the toxin B fragment is responsible for ultimate toxicity; to date no direct evidence exists to document this. Although the isolated light chain and heavy chain region comprising fragment B are nontoxic in animals, this may be because these toxin fragments are unable to be bound and internalized.

Monoclonal antibodies are potentially valuable probes for investigating structure-function relationships of tetanus toxin. A number of laboratories have succeeded in producing either murine (Ahnert-Hilger et al., 1983; Kenimer et al., 1983; Sheppard et al., 1984; Volk et al., 1984) or human (Boyd, et al., 1984, Gigliotti and Insel, 1982, Ichimori et al., 1985, Larrick et al., 1983, Ziegler-Heitbrock et al., 1986) monoclonal antibodies to tetanus toxin or toxoid. Although in naturally occurring situations the amount of tetanus toxin necessary to be lethal is too small to stimulate an immune response, the use of larger amounts of nontoxic tetanus toxoid produces a prominent immunologic response. The toxoid molecule is quite antigenic with evidence for at least 20 epitopes (Volk et al., 1984). Basically two classes of monoclonal antibodies have been produced. One group of monoclonal antibodies do not affect toxicity after interacting with the toxin molecule. These antibodies are potential markers for the toxin action (Habig et al., 1983) or could act as innovative delivery systems. The other group of monoclonal antibodies have the ability to neutralize the action of tetanus toxin. It is this group of neutralizing monoclonal antibodies that can provide insights into structure function relationships of the toxin molecule. The experiments presented here utilize selected neutralizing and nonneutralizing monoclonal antibodies directed against different domains on the tetanus toxin molecule. The production and characterization of these antibodies has been reported (Kenimer et al., 1983).

We have used the experimental system of fetal mouse spinal cord neurons grown in dissociated cell culture. The characteristics of toxin action in this system have been

described (Bergey et al., 1983, 1987). This system offers
distinct advantages over intact animal systems or isolated cell
membrane systems. Intracellular recordings demonstrating the
action produced by tetanus toxin can be obtained following
addition of known concentrations of toxin or toxin-antibody
complexes without requiring toxin transport or diffusion through
dense neuropil. This activity produced by tetanus toxin on
these spinal cord neurons has been demonstrated to result from a
relative reduction of inhibitory synaptic transmission; as such
it is analagous to the actions produced by the toxin in intact
systems while allowing direct observations of neuronal
physiology. To date the studies of neutralization of toxin
activity by monoclonal antibodies have utilized whole animal
bioassays of lethality. Only the reports of Ahnert-Hilger et al.
(1983) utlized an in vitro binding assay of cortical neurons.
The mouse spinal cord neuron culture system allows correlation
between the effects of antibodies on binding of tetanus toxin
with the prevention of the action of the toxin produced by
blockade of synaptic inhibition. Any important insights into
structure function relationships of tetanus toxin require the
ability to separate toxin binding from toxin action. Antibodies
that block binding of toxin neutralize toxin before the toxin has
any membrane or cellular interaction.

The above studies are important investigations into
structure function considerations of tetanus toxin. As
mentioned, however, the actual locus of action of the exquisitely
potent toxin is not known. The ability to antagonize or retard
the action of tetanus toxin with other agents (other than
antibodies) can provide insights into potential sites of action
of the toxin. The neuromuscular blockade produced by tetanus
toxin has been demonstrated to be antagonized by ammmounium
chloride and methylamine hydrocholoride (Simspon, 1983). Simpson
has speculated that this antagonism of action results from the
prevention of internalization of tetanus toxin, the second step
in toxin action (binding, internalization, action). Diptheria
toxin, however, can also be antagonized by ammonium chloride and
chloroquine (Sandvig and Olsnes, 1980; Draper and Simon, 1980).
The mechanism of action of diptheria toxin is much better
understood than tetanus toxin. The antagonism of diptheria toxin
action by chloroquine and ammonium chloride appears to result
from increased pH in lysosomes and reduced effectiveness of
endocytosis. The role of lysosomes and endocytosis in the action
of tetanus toxin is not established. In non-neuronal systems
(e.g. the liver) endocytosis of tetanus toxin has been
demonstrated (Montesano et al., 1982). Tetanus toxin coupled to
gold has been demonstrated in vesicles in spinal cord neurons in
culture (Neale and Bergey, unpublished observations). Whether
such endocytosis is necessary for toxin action or merely an
observed phenomenon is not established, but the ability of
selected agents to antagonize the action of tetanus toxin could
provide insight into the importance of these mechanisms.

Recently investigators in another laboratory at our institution (also working on US Army Medical Research Institute of Infectious Disease, USAMRIID, sponsored research) have found that tetanus toxin prevents the accumulation of cGMP that occurs upon depolarization of PC12 cells in culture (Rogers and Sandberg, unpublished observations). Nonhydrolyzable analogs of cGMP have reversed the toxin-induced blockade of neurotransmitter release. Based on these preliminary data we report investigations on the ability of these cGMP analogues to reverse of the effects of tetanus toxin on the spinal cord neurons in culture. Demonstration of such effects would provide evidence for a potential mechanism of action of tetanus toxin, i.e. that interference with cGMP metabolism affects neurosecretion.

There is a single report that the lectins Concanavalin A and phytohemagglutinin administered intraperitioneally or subcutaneously protected animals against the effects of tetanus toxin (Marconi et al., 1982). The ability of these plant lectins to antagonize tetanus toxin in the spinal cord culture system will be investigated. Previous investigators (Yavin et al., 1981) have found no effect of Concanavalin A on toxin binding to cortical neurons. Since tetanus toxin binds to long chain gangliosides this finding is not surprising. Whether such long-chain gangliosides are the functional receptors for tetanus toxin is controversial; antagonism of toxin action by plant lectins that bind to sugar moieties would provide further evidence for important binding sites for tetanus toxin independent of the long-chain gangliosides.

METHODS

Culture Techniques

Cultures of fetal mouse spinal cord neurons were prepared as described in detail previously by Ransom et al. (1977). Spinal cords were removed from 13-14 day old fetal mice and then pretreated with trypsin before mechanical dissociation. The cells were then plated on collagen-coated 35 mm plastic culture dishes. The culture medium was Eagle's minimal essential medium (MEM) supplemented with glucose (final concentration 30 mM and bicarbonate (final concentration 44 mM). Cultures were grown and maintained at 35 C in 10% CO_2. During the first 24 hours both 10% fetal calf serum and 10% horse serum (HS) were included in the culture medium. After this time only 5% HS was included and 1% N3 solution (Romijn, 1982) was added. The antimetabolite 5-fluoro-2-deoxyuridine was used for a 24-h period after day 6 to limit the growth of nonneuronal cells. Cultures were maintained with biweekly subtotal changes of medium for 4-8 weeks at which

time they were used for experiments.

Tetanus Toxin

Homogeneous tetanus toxin was prepared from sterile filtrates of <u>Clostridium tetani</u> cultures as previously described by Ledley et al. (1977). The toxin has about 2 X 10^7 mouse lethal doses (MLD) per milligram of toxin protein. An MLD is defined as the least amount of toxin that will kill a 15-18 g mouse within 96 h following subcutaneous injection into the inguinal fold region.

Radiolabeled toxin was prepared using ^{125}I-Bolton-Hunter reagent (2,000 Ci/mmol, New England Nuclear) as described by Bolton and Hunter (1973) and An der Lan et al. (1980). The specific activity of the ^{125}I-tetanus toxin was about 2 mCi/mg protein and at least 80% of the biotoxicity was retained.

Antitetanus Antibodies

Monoclonal antibodies specific for epitopes on fragment B (light and heavy chain regions) and Fragment C were prepared by fusion of P3X63Ag8 BALB/c myeloma cells with spleen cells from BALB/c mice immunized with tetanus toxoid or fragment B as described in detail previously (Kenimer et al., 1983). From the library of 14 monoclonal antibodies produced against various toxin epitopes, 4 monoclonal antibodies were selected for experimentation based on neutralization studies in whole animals (Kenimer et al., 1983). In this paper the monoclonal antibodies will be identified using the same nomenclature as in the original report. Two monoclonal antibodies directed against the C fragment of tetanus toxin were used, one (18.1.7) shown to have a neutralization titer of 0.3 U/ml, the other (18.2.12.6) having no detectable neutralization titer (<0.001). Two monoclonal antibodies directed against the B fragment of tetanus toxin were utilized. One (21.76.10) was directed against the heavy chain portion of the B fragment and had a high neutralization titer of 3.0 U/ml. The other (21.18.1) was directed against a site on the light chain of the B fragment and had no detectable neutralization titer (<0.001). Figure 1 illustrates the various monoclonal antibodies and their binding domains.

Horse serum (Gibco Laboratories) and mouse serum (supplied by W. Habig) were used as sources of polyclonal antibodies.

Toxin Binding Studies

Experiments were performed to determine tha effects of respective monoclonal antibodies on the binding of tetanus toxin to spinal cord neurons. To determine the specificity of toxin binding, experiments were done where a 10-fold excess (relative to labeled toxin) of unlabeled toxin was applied to cultures prior to the addition of ^{125}I-labelled tetanus toxin.

^{125}I-tetanus toxin was preincubated in culture media with excess monoclonal antibody or polyclonal antibody for one hour prior to the addition to spinal cord cultures. The number of counts added was determined by an assay of equal volume (2cc) of the antibody-toxin mixture. Cultures were incubated with the various antibody-toxin combinations for 1 hour at 35 C. Cultures were then washed 3 times in MEM with 1% FCS (which contains proteins but no antitetanus toxin antibodies). For each assay, 5 cultures were used. Following the washes, cultures were harvested and counts of culture associated ^{125}I-tetanus toxin were made.

Electrophysiology

Cultures were selected for electrophysiological studies after growing for 4 to 8 weeks. Cultures were washed 3x in MEM with 1% fetal calf serum to remove all horse serum (horse serum contains antitetanus antibodies at high titers). After washing no detectable horse serum is present (i.e. <0.001 U). Cultures were then placed on a heated (33-34 C.), CO_2-gassed (10%) stage of an inverted phase-contrast microscope. Intracellular recordings were made under direct vision using microelectrodes filled with 4 M potassium acetate at neutral pH and pulled to yield resistances of 30-50 MOhm. A conventional bridge circuit was used for recordings in conjunction with a storage oscilloscope and continuous chart recorder.

A dilution of purified tetanus toxin (400 ng/ml) was added to the cultures in 500 ul aliquots to yield a final concentration of toxin of 100 ng/ml. In experiments using antibody preparations, excess antibody was added to stock tetanus toxin (400 ng/ml) and preincubated for 1 hour at 35 C. before adding a 500 ul aliquot to the spinal cord cultures. The 200 ng of toxin present in the 2 cc of culture media represents about 2000 mouse lethal doses. Intracellular recordings were begun within 5 minutes after the addition of the toxin preparations. Multiple neurons were sampled over the following 3 hours to document the presence or absence of convulsant activity.

Tests of Potential Antagonists of Tetanus Toxin

Experiments were performed to determine whether ammonium chloride (5-20 mM final concentration), chloroquine (0.01-0.1 uM final concentration) or methylamine HCl (10 mM final concentration) reduced the time to onset of convulsant activity produced by tetanus toxin.

Initial experiment were performed by mixing the respective agents in culture media, changing the culture media to include the agent. Control intracellular recordings were made from 10-20 spinal neurons at 30 minutes to 6 hours after the addition of the new media to determine whether the agent itself had any affect on the neuronal behavior. Cultures were also observed under a phase microscope to assay for any obvious morphological changes.

If no deleterious morphological changes were seen (i.e. cytotoxicity) and no overt changes in the physiological behavior of the neurons was observed then comparisions were made to see if the selected agent retarded or prevented the onset of action of tetanus toxin as measured by the appearance of paroxysmal depolarizing events. Spinal cord cultures were preincubated for hours in the experimental media and the time to onset of convulsant activity following addition of tetanus toxin (final concentration) was noted. This was compared with the time to onset of convulsant activity produced in control cultures. Five to 10 assays of onset of PDE were performed for each experimental condition.

Experiments with Concanavalin A were conducted in a similar fashion to those above using spinal cord cultures preincubated in 50-400 ug/ml Concanvalin A (Sigma) for 1-6 hours. Experiments with FITC-labeled Con A have shown that binding occurs to both neuronal and nonneuronal cells in this system.

To assay whether the cGMP analogue, 8 bromoguanasine 3'-5'-cyclic monophosphate (Sigma, 8-bromo GMP), could reverse the tetanus toxin produced reductions in neurotransmitter release, 8 bromo cGMP was added (final concentration 0.1-1.0 mM) to spinal cord cultures previously incubated for 24 hours in 100 ng/ml tetanus toxin. Single neurons were sampled using intracellular electrodes to assay for spontaneous electrical activity. In addition pairs of potentially connected neurons were sampled to determine whether any functional synaptic connections were present. Other experiments were performed during the onset of convulsant activity produced by tetanus toxin. Following addition of tetanus toxin (100 ng/ml final concentration), pairs of monosynaptically (excitatory) connected neurons were identified (a monosynaptic connection was operationally defined as a connection with less than 5 mS latency and low failure rate). Recordings of synaptic cell pairs were performed in media containing 6 mM Ca^{2+} and 6 mM Mg^{2+} to reduce spontaneous synaptic

activity yet allow evoked synaptic activity. Only after a stable recording period had been established with no drift in resting membrane potential and the effects of tetanus toxin began to be evident as manifested by the gradual onset of the reduction in the monosynaptic postsynaptic potential (stimulating the presynaptic neuron at 1/sec) was the 8 bromo-cGMP added. The size of the evoked PSP was monitored continuously.

RESULTS

Effects of Antibodies on Binding of Tetanus Toxin

The selected monoclonal antibodies and their binding domains on the tetanus toxin molecule (as determined previously, Kenimer et al., 1983) are illustrated (Fig. 1). The ability of the respective monoclonal and polyclonal antibodies to block ^{125}I-tetanus toxin binding is shown in Table 1. Experiments using unlabeled tetanus toxin prior to the addition of labeled toxin demonstrated that 78% of the tetanus toxin was specifically bound.

In control cultures with no antitetanus antibodies present, 16% of the total counts of the labeled toxin were bound. The equine polyclonal tetanus antitoxin (TAT) and the mouse polyclonal antibody preparations were the most effective in reducing the amount of toxin bound; in both instances less than 1% of the added toxin was bound after preincubation with the two antibody preparations. One of the monoclonal antibodies directed against an epitope on the C fragment of the toxin, antibody 18.1.7, was also quite effective in preventing toxin binding, with only 1.5% of counts being bound. In contrast, another monoclonal antibody directed against a region of the C-fragment, antibody 18.2.12.6, did not reduce the amount of toxin bound when compared to controls without antibodies. Similarly, one of the monoclonal antibodies directed against an epitope on the B-fragment, antibody 21.18.1, did not reduce toxin binding. A small reduction (25% compared to controls) of toxin binding was seen with the other monoclonal antibody (21.76.10) directed against an epitope on the heavy chain of the B fragment.

Effects of Antibodies on Convulsant Activity Produced by Tetanus Toxin

Figure 2 illustrates the paroxysmal activity produced by tetanus toxin. Previous characterization of the action of tetanus toxin on spinal cord neurons in culture (Bergey et al., 1983, 1987) has demonstrated that following the addition of toxin there is a dose dependent latent period with no observable change seen with intracellular recordings of spontaneous electrical

activity. Presumably during this latent period the toxin is binding to the neuronal membrane and undergoing a subsequent membrane interaction or internalization. Following the latent period increased excitatory activity is seen. Paroxysmal depolarizing events (PDE), abrupt 5-20 mV depolarizations with resultant triggered action potentials, lasting several hundred to thousand milliseconds are produced. This paroxysmal activity parallels the reduction and disappearance of spontaneous and evoked inhibitory potentials as the toxin progressively blocks presynaptic release of neurotransmitter.

With concentrations of tetanus toxin of 100 ng/ml tetanus-PDE become established typically after 40-70 minutes although further evolution to a more regular pattern of bursting may occur subsequently (Fig. 2, 3). All spinal cord neurons that are destined to develop tetanus-PDE will do so by 45-90 minutes after the addition of 100 ng/ml at 33-34 C. Because of the abundant synaptic connections in the spinal cord cultures, 90-95% of the spinal cord neurons will show PDE after the addition of toxin (Bergey et al., 1983). Those few neurons that do not develop tetanus-PDE usually have few observed spontaneous excitatory postsynaptic potentials (EPSPs); presumably an insufficient excitatory network is present in these instances to produce the synaptically mediated PDE.

Intracellular recordings from spinal cord neurons were performed on spinal cord neurons up to 3 hours after the addition of tetanus toxin (100 ng/ml) or the various toxin-antibody mixtures. At 3 hours, concentrations of tetanus toxin as low as 10^{-9} g/ml would be expected to produce convulsant activity at 33-34 C. (Bergey et al., 1983).

The ability of the various antibody preparations to prevent the convulsant activity produced by tetanus toxin in these spinal cord cultures is illustrated in Figure 3. At least three experiments were performed with each antibody-toxin combination. In each experiment, recordings from at least 15 spinal cord neurons were obtained to determine the neutralizing ability of the antibodies. In instances of neutralization, sampling was continued until 3 hours after the addition of the toxin-antibody mixture. The polyclonal mouse antibody and the equine TAT (not shown) neutralized the effect of tetanus toxin; no change in the control pattern of spontaneous electrical activity was seen. One of the monoclonal antibody directed against fragment C (18.1.7) was also effective in preventing tetanus-PDE; this was the antibody that had been demonstrated to markedly reduce toxin binding (Table 1). The other monoclonal antibody directed against the C fragment (18.2.12.6) did not prevent the development of PDE; this antibody did not reduce toxin binding in the previous studies. Antibody 21.18.1, directed against an epitope on the light chain portion of the B fragment, did not block the development of paroxysmal activity produced by tetanus

toxin. In contrast, antibody 21.76.10, an antibody directed against a region on the heavy chain of the B fragment, prevented the development of tetanus-PDE. This antibody (21.76.10) was one that did not prevent the binding of tetanus toxin.

Effects of Potential Antagonists of Tetanus Toxin Activity

Chloroquine was nonspecifically toxic to both neuronal and non-neuronal neurons in culture. Even after several hours, spinal cord neurons were markedly vacuclated, particularly at the higher concentrations and stable intracellular recordings could not be obtained. With longer incubation periods (up to 24 hours) over neuronal cell death and vacuolation of the non-neuronal background cells was evidenct.

Ammonium chloride (5-10 mM) produced paroxysmal depolarizing activity itself independent of the addition of tetanus toxin. This result made it impossible to assay for the onset of convulsant activity produced by tetanus toxin since the control situation was altered by the addition of ammonium chloride.

The addition of methylamine HCl (10 mM) produced no changes in the background spontaneous electrical activity of the spinal cord cultures. The average time to onset of tetanus-PDE (100 ng/ml tetanus toxin) was 54.8 + 18.4 without methylamine and 61 + 17.8 (sd) with methylamine. These differences were not significant.

Addition of Concanavalin A (50-400 ug/ml) resulted in a marked diminution of spontaneous electrical activity and synaptic potentials. Convulsant activity could not be produced by tetanus toxin after the preincubation of the cells with Con A.

The experiments with 8-bromo cGMP did not demonstrate the ability of this analogue to reverse the synaptic blockade produced by tetanus toxin after 24 hours (42 cells; 32 potential cell pairs). Because the possibility existed that this period of time of toxin exposure resulted in large amounts of internalized toxin that possibly could not be reversed, the studies of monosynaptic cell pairs were done. Following the identification of monosynaptically connected (excitatory or inhibitory) cell pairs, 8-bromo cGMP was added and the amplitude of the PSP was monitored. Addition of the CGMP analogue was at a time when the PSP was beginning to be reduced in size indicating that the toxin had begun to act. The PSP was then monitored for 20-30 minutes. A total of 6 monosynaptic connections were successfully held for this period of time. In no instance did the 8-bromo cGMP reverse the progressive diminution in PSP size.

DISCUSSION

Of the fourteen monoclonal antibodies originally produced by Kenimer et al. (1983) two are directed against Fragment C of the toxin molecule, the portion containing the binding subunit. Both of these antibodies were examined in the spinal cord cultures reported here. One of the antibodies (18.1.7) is neutralizing in the spinal cord culture system as it is _in vivo_; this antibody markedly reduces toxin binding. Since binding of tetanus toxin is the first step in toxin action, it is not surprizing that blockade of binding would neutralize the action of the toxin. The other antibody directed against Fragment C (18.2.12.6) does not neutralize the convulsant action of tetanus toxin on the cultured neurons; as expected it also does not reduce toxin binding to the cultures.

Two of the monoclonal antibodies studied here are directed against fragment B. The monoclonal antibody directed against a light chain domain (21.18.1) is nonneutralizing in the spinal cord culture system. Of the nine clones directed against the light chain produced by Kenimer et al. (1983) none have significant neutralization titers. The monoclonal directed against the heavy chain portion of fragment B (21.76.10) is able to effectively neutralize the action of tetanus toxin on the spinal cord neurons in culture. The ability of an antibody directed against the heavy chain of fragment B to neutralize toxin activity may be due to several potential mechanisms. This may indicate that the active domain of the toxin resides on the heavy chain portion of fragment B. Alternatively the antibody, by binding to the heavy chain, may have prevented translocation or internalization of the toxin so that the light chain could not reach its site of action. A third possibility is that the antibody-fragment B heavy chain interaction may have produced a conformational change that prevented the light chain from retaining activity. These data nevertheless provide further evidence for fragment B containing the active domain.

No laboratories to date have reported a neutralizing monoclonal antibody directed against the light chain of tetanus toxin. Of the six neutralizing monoclonal antibodies reported by Ahnert-Hilger et al. (1983) only a single antibody directed against fragment B was reported and this antibody neutralized toxin binding with cortical membranes suggesting an interference with the function of the binding region on fragment C. Of the 57 hybridomas produced by Volk et al. (1984) only one bound preferentially to the light chain. All neutralizing antibodies bound to the heavy chain, including 6 directed to the heavy chain portion contained on fragment B (fragment I_{bc} using the terminology of Bizzini et al., 1977). Volk et al. (1984) provide no data as to the ability of these neutralizing antibodies to antagonize binding of tetanus toxin to neuronal membranes. As demonstrated by the anti-fragment B antibody of Ahnert-Hilger et

al. (1983) an antibody directed against the fragment B heavy
chain can antagonize binding so binding studies must be included
before one can conclude that the anti-fragment B antibody is
acting by specifically neutralizing fragment B action. Although
a small reduction in labelled toxin binding (about 25% compared
to control) was seen with the neutralizing antibody directed
against fragment B here (21.76.10) this reduction would only be
expected to delay the onset of tetanus produced convulsant
activity by at most about 15-20 minutes (based on dose-response
data, Bergey et al., 1983), still well within the assay time.
Therefore the neutralization produced by antibody 21.76.10
results from its interaction with fragment B.

Several laboratories have succeeded in producing human
monoclonal antibodies against tetanus toxin (Boyd, et al., 1984,
Gigliotti and Insel, 1982, Ichimori et al., 1985, Larrick et al.,
1983, Ziegler-Heitbrock et al., 1986). Gigliotti and Insel
(1982) describe a protective human monoclonal antibody directed
against fragment B of the toxin molecule, but no reactivity was
seen against purified tetanus toxin suggesting that the antibody
may require the conformational structure present in whole toxin
and is not directed against a distinct locus on either the light
or heavy chain of fragment B. Ziegler-Heitbrock et al. (1986)
report protection produced by a combination of two human
monoclonal antibodies, one against a domain on fragment C, and
another against the heavy chain portion of fragment B; neither
monoclonal antibody by itself was protective. Indeed the
therapeutic use of monoclonal antibodies appears to be limited by
the fact that to date they are less effective in neutralization
than polyclonal antibody preparations or combinations of
monoclonal antibodies (Mizuguchi et al., 1982, 1984; Volk et al.,
1984).

The above results confirm that antibodies that prevent toxin
binding to neurons neutralize toxin action. The ability,
however, of a monoclonal antibody directed against the heavy
chain portion of the B fragment to neutralize toxin action while
not preventing binding of tetanus toxin to the cultured spinal
cord neurons indicates that while binding of tetanus toxin to
neurons is a necessary prerequesite, it is not sufficient for
toxin action. These findings indicate that the nonbinding
fragment B of tetanus toxin is important for toxin action.
Whether the ability of tetanus toxin to ultimately block
neurotransmitter release resides on the light or heavy chain of
the B fragment is not definitely estabished. Similarly whether
the neutralizing antibody to fragment B utilized here acts by
preventing toxin internalization or acts at the as yet
undetermined cellular site of action remains to be determined.
Selected monoclonal antibodies against domains on fragment B of
the toxin molecule can be potentially valuable probes of the
sites of toxin

The results to date on potential antagonists of tetanus toxin have not been as fruitful as the monoclonal antibody studies. Chloramine was toxic to the neurons in culture. Ammonium chloride produced convulsant activity itself, making our assay of tetanus toxin action difficult to perform. Presumably the ammonium chloride is antagonizing inhibitory transmission. It may still be possible to test the ability of ammonium chloride to antagonize tetanus toxin but it will be necessary to use the blockade of all synaptic transmission as the measure of toxin action (a later effect after the convulsant period) and less than complete effects may not be able to be determined. The methylamine experiments did not show any difference in time to onset of tetanus-PDE. It may very well be that the lysosomal compartment (at least the pH) is not critical for toxin action.

Concanavalin A prevented tetanus toxin from producing paroxysmal activity in the spinal cord neurons in culture, but this resulted from a dramatic reduction in spontaneous synaptic activity. The ability of Con A to affect both transmitter release (Grasso et al., 1978) and postsynaptic receptor function (Messing et al., 1984; Mathers and Usherwood, 1976) has been previously reported. Full characterization of the Con A effect in these cultures was not done since this would not have provided additional insights into tetanus toxin action. Indeed the Con A effect was almost certainly a masking of toxin action (i.e. it was not apparent in the absence of synaptic activity) rather than prevention. These results do not explain how in whole animals, peripherally injected Con A would be protective (Marconi et al., 1982).

The experiments with the cGMP analogues failed to reveal reversal of the effects of tetanus toxin. This does not absolutely rule out reversal because of the nature of the assay system. The experiments testing neurons exposed for 24 hours may have had so much internalized toxin that reversal could not be produced; smaller amounts of toxin might be reversed. The dynamic observations of monosynaptic PSP amplitudes may have required more internalized cGMP. Additional experiments using smaller amounts of toxin may provide evidence for some reversal.

The monoclonal antibodies (specifically the anti-B fragment neutralizing antibody) provides the best tool available to date to probe toxin activity. It now remains to combine these antibody probes with tetanus toxin labeled so as to be identified ultrastructurally so that subcellular compartments of action can be studied in conjunction with the physiological actions of the toxin.

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Table 1. Effects of Antibodies on Tetanus Toxin Binding

Toxin + Antibody Percent Bound	Counts Added	Counts Bound
Control - No Ab 16	162,524	31,013 + 1377
18.1.7 (Anti-C) 1.5	190,700	2,961 + 243
18.2.12.6 (Anti-C) 18	191,263	34,673 + 854
21.76.10 (Anti-B) 12	189,739	23,983 + 1626
21.18.1 (Anti-B) 17	197,083	32,701 + 1270
Equine TAT 0.1	200,971	156 + 43
Mouse polyclonal Ab 0.9	215,594	1,700 + 104
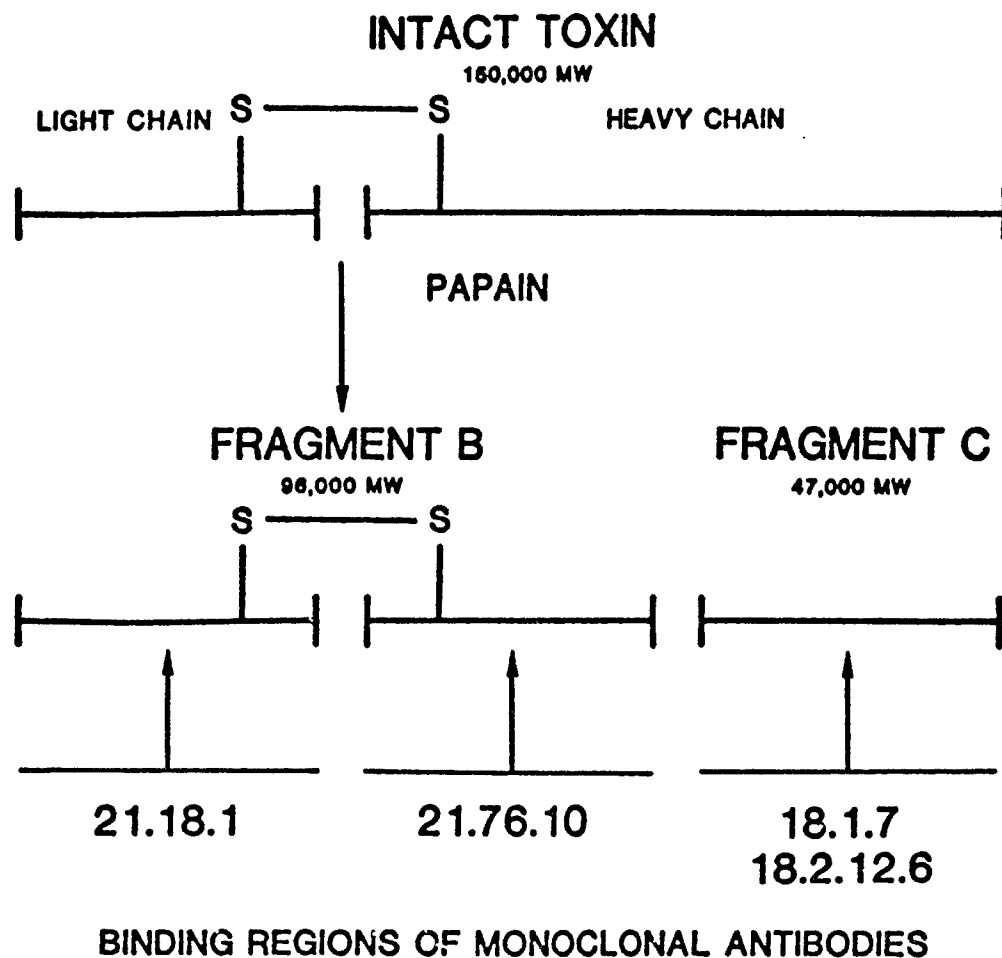
Figure 1.

The binding domains of the four monoclonal antibodies used for experiments are shown, as determined previously (Kenimer et al., 1983) by ELISA reactivity.
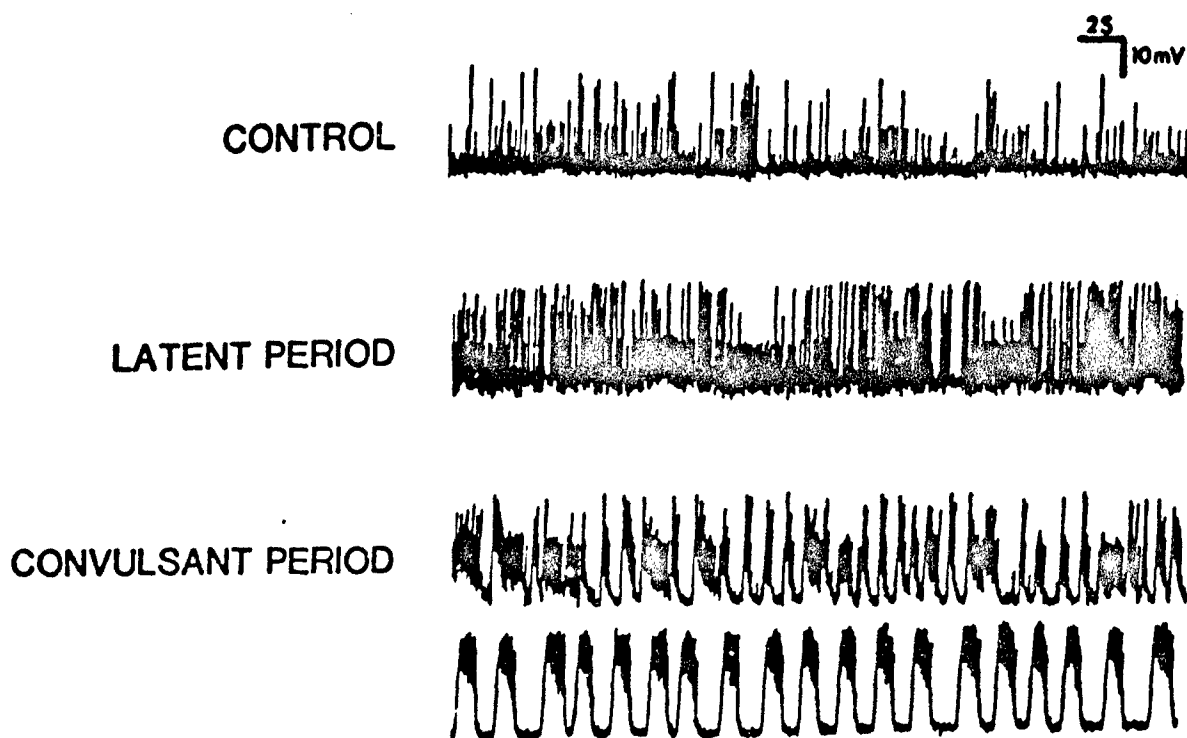
CONTROL

LATENT PERIOD

CONVULSANT PERIOD

Figure 2.

The stages of action of tetanus toxin on mouse spinal cord
neurons in culture are illustrated by representative chart
records from intracellular recordings from a spinal cord neuron
during each illustrated period. Recordings from spinal cord
neurons in control media reveal an irregular pattern of
spontaneous action potentials and excitatory and inhibitory
potentials. Following the addition of tetanus toxin to the media
(final concentration 100 ng/ml) there is a dose-dependent and
temperature dependent latent period (typically 40-70 minutes with
100 ng/ml of toxin at 33 C). During this latent period little or
no change in the spontaneous activity of the neurons is seen.
Following the latent period, increased excitatory activity is
seen as manifest by paroxysmal depolarizing events (PDE) with
associated triggered action potentials (convulsant period). This
paroxysmal activity can at times become quite regular and
rhythmic as shown in the second record in the convulsant period.
The amplitudes of the action potentials in these and other chart
records are attenuated due to the frequncy response of the
penwriter
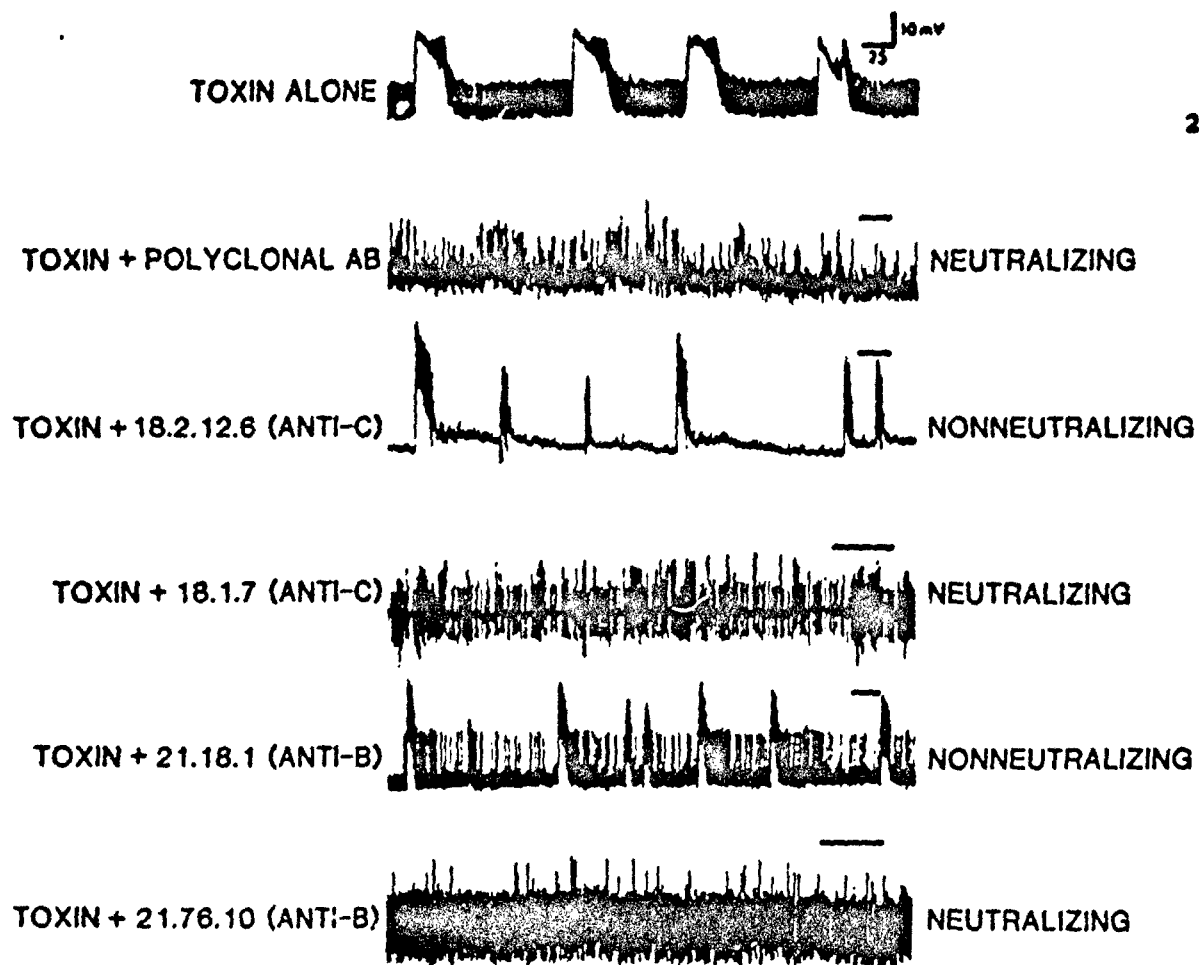
Figure 3.

Representative chart records from spinal cord neurons 1 1/2 to 3 hours after exposure to 100 ng/ml of tetanus toxin preincubated with selected antibodies. Neutralization is the ability of the antibody to prevent the development of the convulsant activity produced by tetanus toxin as manifest by the prominent, often rhythmic parosysmal depolarizing events (PDE).

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